STUDY OF THE CYTOTOXIC EFFECT OF ORTHOPEDIC MATERIALS BASED ON ETHYLENE-VINYL ACETATE ON CELL CULTURES

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Measuring the potentially adverse effects of materials on the human body is vital and essential for their use, including medical applications. One of the properties that need to be known is the cytotoxic effect of the materials to avoid local and systemic adverse effects. It is very important that scientists and medical professionals are aware of the effects of materials on the human body. This study also summarizes the data on the morphology, chemical composition, and the effect of ethylene-vinyl acetate (EVA) orthopaedic materials on cytotoxicity. Qualitative and quantitative cytotoxicity analysis was performed using fluorescent microscopy and metabolic cell toxicity assay. The results of the studies demonstrated low in vitro cytotoxicity and high biocompatibility. These results may serve as the basis for a toxicological study of orthopaedic materials to prevent severe side effects.

KEYWORDS

material, qualitative analysis, electron microscope, cytotoxicity, biocompatibility, medical devices, ethylene-vinyl acetate

1. INTRODUCTION

Medical devices have a direct impact on human health and are subject to the requirements of the regulatory and legal frameworks in the field of health care. This refers to the characteristics and properties of materials, including chemical, toxicological, physical, electrical, morphological, and mechanical properties. According to the recommendations of the ISO 10993 [ISO 10993-1:2018] series of standards, a systematic approach to the study of the biological evaluation of medical devices is required.

Orthopaedic products are part of a huge medical products industry; their purpose is to improve the patient's life by repairing or treating injuries [Wang 2021, Yang 2021]. Shoes with orthopaedic insoles (Fig. 1) are widely used in the treatment and prevention of many diseases, people need to solve various foot problems [Nouman 2019, Mariyam 2021, Ahmed 2021]. The most progressive today are individually made insoles [Ahmed 2021, Girard 2020], based on three-dimensional scanning of the patient's foot [Chatzistergos 2020]. Theoretically, different materials are suitable for the manufacture of foot orthoses and orthopaedic shoes, the limitations are in biocompatibility, cost and durability [Paul 2021]. Ethylene Vinyl Acetate (EVA) copolymer is one of the most commonly used materials in shoe midsole construction due to its durability, flexibility and thermal viscosity [Gerrard 2020]. Because these products are in direct contact with tissues and cells of the human body, they not only require good physical and chemical properties but must also have good biocompatibility. Among biocompatibility tests, cytotoxicity is preferred as an important indicator for the evaluation of medical devices because it is simple, fast, highly sensitive and can save people from toxicity. Thus, in order to meet regulatory requirements and be used in medical applications, the cytotoxicity of EVA polymeric materials must be studying in detail. Medical professionals and patients lack the scientific evidence to determine and use the ideal material for orthopaedic insoles [Zaborowski 2007, Macala 2009, Panda 2013, 2014, 2016, 2019 and 2021, Valicek 2016 and 2017, Balara 2018, Duplakova 2018, Pandova 2018].

It is critical that clinicians prescribing foot orthoses have reliable data on the biological performance of orthotic materials to inform clinical decision making. This is especially true given the forecast for rapid growth in the orthotic foot industry [Karkalica 2020]. If human health is expected to be exposed to any particular toxic substance, careful quantification of each of these substances must be carried out.

The object of this study is to evaluate the in-vitro cytotoxicity profile of polymeric materials of ethylene-vinyl acetate that differ in chemical composition.

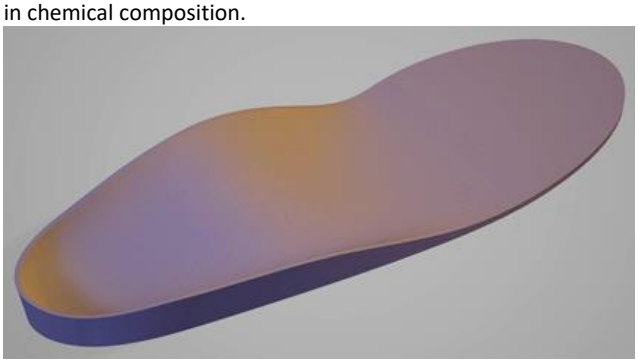


Figure 1. 3D model of free-form orthopedic insoles

2. MATERIALS AND METHODS

The most common materials for orthopedic insole are EVA (26%), Poron (13%), Plastazote (11%), PU (9%), PPT (5%) and others [Ramli 2018]. EVA material has the lowest stress concentration compared to other materials such as isoprene, butyl rubber, polyurethane, neoprene, foam rubber and micro cellular rubber. EVA provides greater elasticity than other materials [Haris 2021]. The EVA compound is a highly elastic copolymer of ethylene and vinyl acetate, available in vinyl acetate levels typically in the range of 10 to 40 wt% [Sood 2021].

Its structure consists of both elastic and transitional segments, which respectively give the EVA products elasticity and variability of shape; from soft (at high temperatures) to hard (at low temperatures) [Kwon 2021]. Various additives are used to prepare blends, including calcium carbonate, silicon dioxide, zinc oxide, stearic acid, dioctyl phthalate, azodicarboamide, and dicumyl peroxide [Gama 2021]. The cytotoxicity test is the most common biological evaluation and screening method, using tissue cells in vitro to monitor the growth, reproduction, and morphological effects of medical devices [Junior 2021]. Cytotoxicity is one of the most important methods of biological

evaluation, as it has a number of advantages along with the desired and required parameters.

Cell culture studies determine cell lysis, cell growth retardation, and othereffects due to medical devices, materials, and/or extracts thereof. Methods for the study of cytotoxicity are described in ISO 10993-5. Due to the variety of medical devices, the variability of the body's environment and the complexity of the interaction between the body and medical devices, a single method of assessment or cytotoxicity assessment system has not yet been developed.

Three types of cytotoxicity tests are specified in ISO 10993-5: extract tests, direct contact tests and indirect contact tests (including agar coverage and filter diffusion tests).

In general, the extracted test is suitable for determining the toxicity of soluble substances in medical devices and is generally consistent with the results of toxicity tests in animals. The direct contact method is the most sensitive to test for cytotoxicity of medical devices; medical devices can be measured even with low cytotoxicity. The agar overlay assay is suitable for medical devices that have high toxicity and volumetric filtration, while the molecular filtration method is suitable for evaluating the biocompatibility of low molecular weight toxic components of medical devices. Gao et al found a good correlation between the test for direct and indirect contact and less correlation between the test for the extract and the other two tests. Samples of EVA polymeric materials were obtained for the study, which were marked with numerical identifiers from 1 to 12 (Fig. 2).

Figure 2. Materials selected for EVA cytotoxicity studies

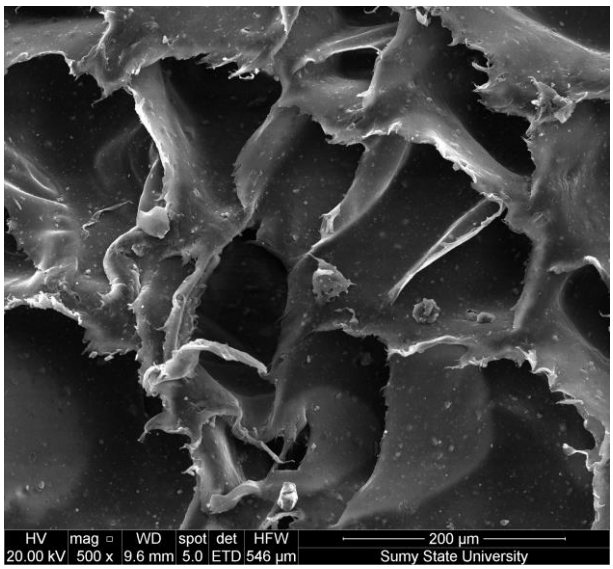
Materials of various shapes and sizes were studied unchanged in cytotoxicity studies. One of the sides of the solid specimen was flat. The EVA composite was analyzed by scanning electron microscope (SEM) using a Quanta 400 FEG ESEM/EDAX Genesis X4M. The EVA composite was analyzed by scanning electron microscope (SEM) using a Quanta 400 FEG ESEM/EDAX Genesis X4M. The experiment used a culture of mesenchymal stem cells (MSCs), which was obtained from the umbilical cord (protocol of the ethics committee and permission to collect material №7 / 12 from 25.03.2020). All reagents for the experiment were obtained from Gibco® (Gaithersburg, MD, USA).

Mesenchymal Stem Cells (MSCs) were grown in 75 cm2 cell culture flasks under standard conditions for culturing humidified air containing 5% CO2 at 37 °C with renewal every 2-3 days. We used a modified Dulbecco of medium and nutrients Eagle F-12 (DMEM / F-12) with L-glutamine containing 100 units/ml penicillin, 100 µg / ml streptomycin, 2.5 µg / ml amphotericin B and 10% fetal bovine serum. The samples were sterilized by immersing them in 70% ethanol solution for 24 hours with simultaneous exposure to ultraviolet light. After sterilization, the samples were placed in a separate well of a 24-well plate for culturing cells and immersed in DMEM overnight to neutralize ethanol residues. MSCs were seeded in each sample and in wells without samples (as a positive control) with a cell density of 10^4 cells per well. The adhesion and proliferation of the cells on the samples was assessed by colorimetric analysis of Resazurin as follows: the plates were incubated for 8 h at 37 °C in the dark, and then medium was added. In the next step, the medium was transferred to another 96-well plate, and the absorbance was measured with a Multiskan FC plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 570 and 595 nm. The number of cells was determined at different intervals: 1, 3 and 7 days. All experiments were repeated 3 times. Statistical analysis of the results was based on one-way analysis of variance ANOVA (GraphPad Prism 8.0 software), and a p value less than 0.05 was considered statistically significant.

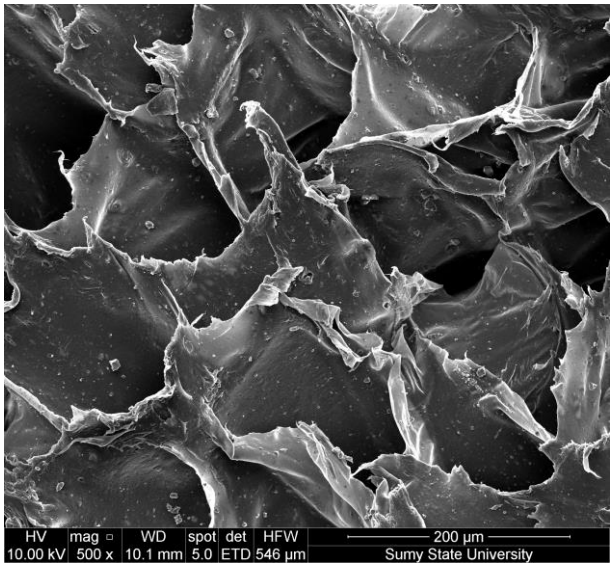
To determine the distribution of cells on the surface of the sample was performed fluorescence microscopy on the 7th day after the experiment with cell culture. The samples were washed with 1 × PBS for 1 min without shaking. The samples were then incubated with 1: 35,000 4 ′, 6-diamidine-2′-phenyldinol dihydrochloride (DAPI, Roche) in PBS for 2 min in the dark followed by washing in 1 × PBS for 1 min without shaking. The samples were then placed on a glass slide and analyzed using a fluorescence microscope (Axio Imager A1 microscope, Carl Zeiss) in a DAPI (358 nm) channel.

3. RESEARCH METHODOLOGY

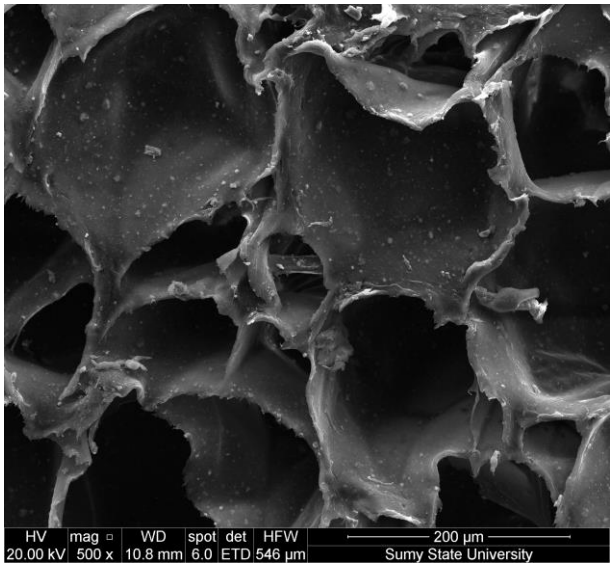
Part of assessing the biological properties of medical devices is to obtain sufficient quantitative information to assess the suitability of all materials for their intended purpose in a ready-to-use device. The results of the study of the morphology and surface structure of the samples from the scanning electron microscopy (SEO-SEM Inspect S50-B) are shown in Fig. 3, revealing a uniform distribution of cells that are practically identical in size.



a) Sample 1



b) Sample 2



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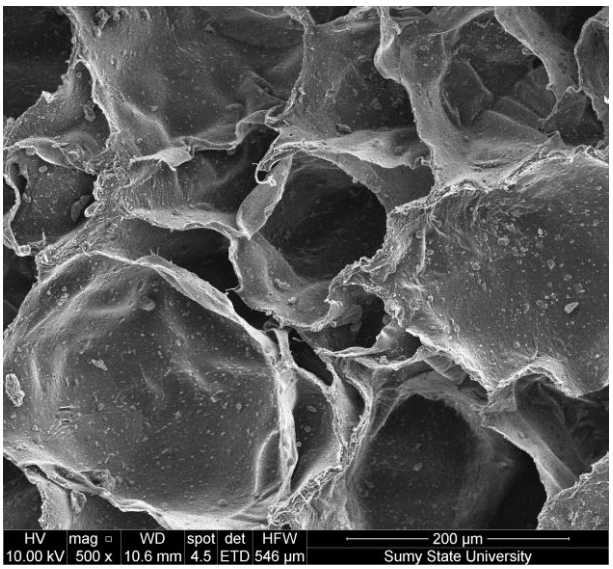

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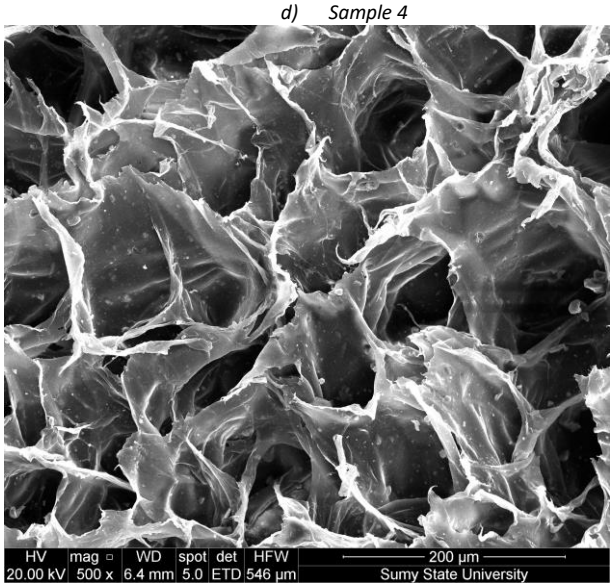
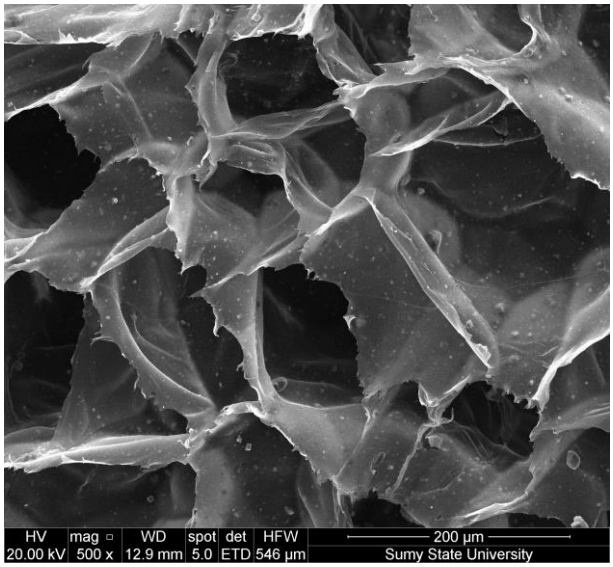
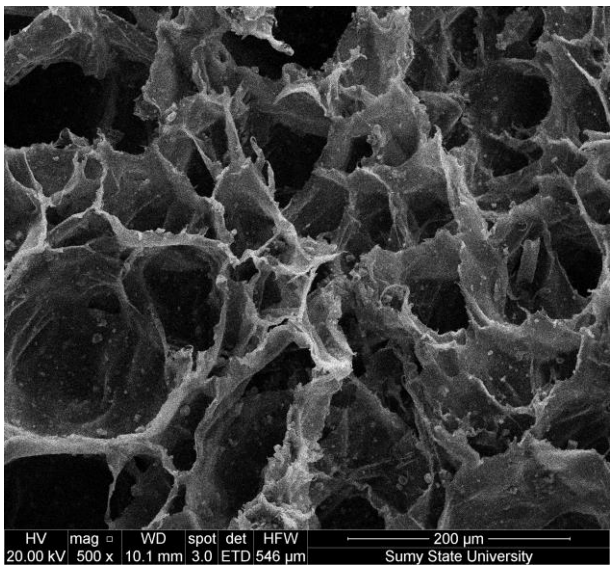


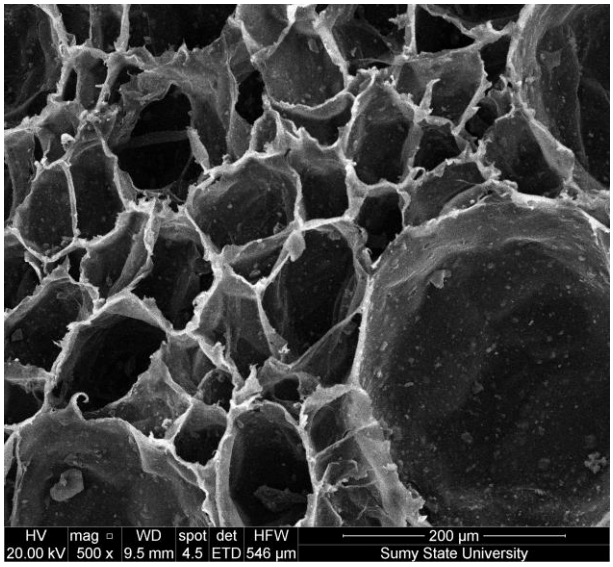
e) Sample 5



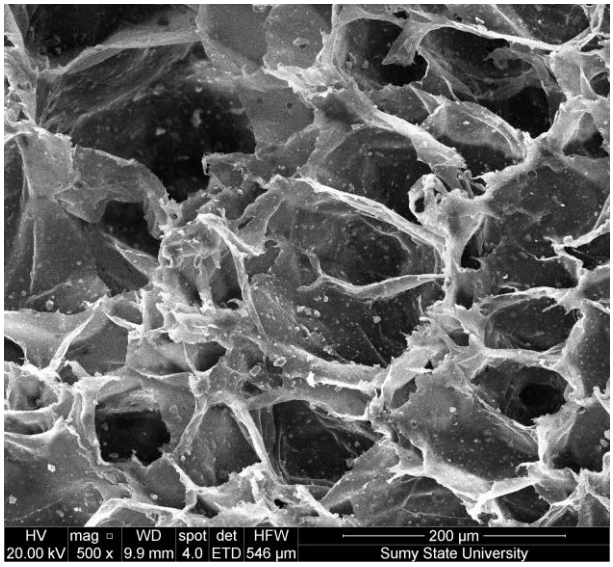
f) Sample 6



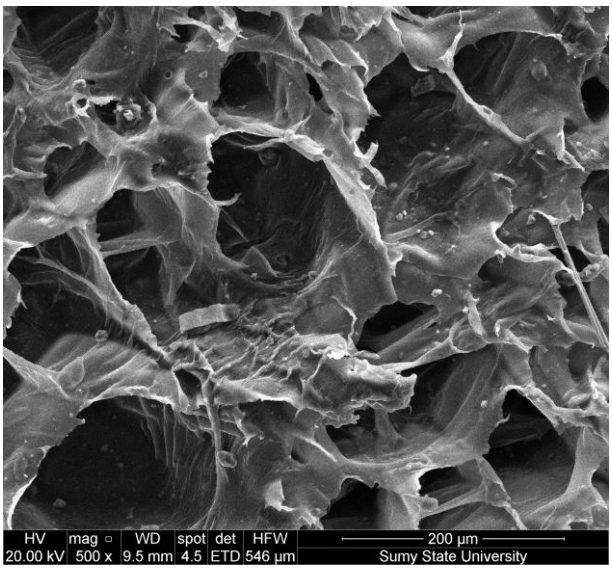
g) Sample 7



h) Sample 8



i) Sample 9

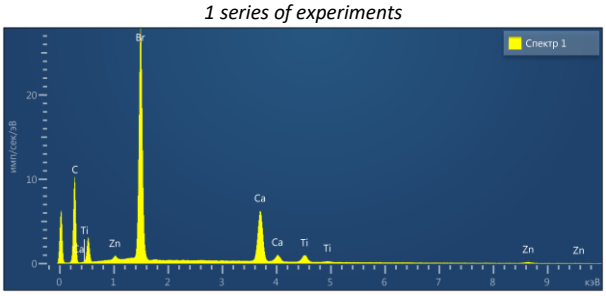


j) Sample 10

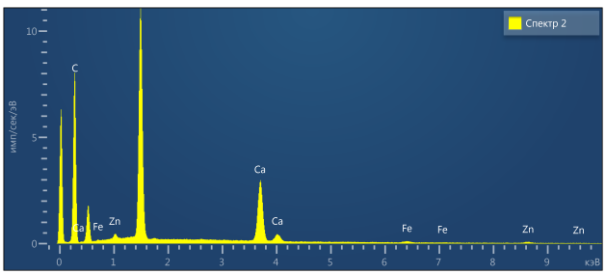
Figure 3. SEM images of the mm EVA polymer sample

The results of energy-dispersive analysis in the study of objects in a scanning electron microscope using an AZtecOne spectrometer with an X-MaxN20 detector (manufactured by Oxford Instruments plc) are presented in Fig. 4 and Table 1.

1 series of experiments



a) Sample 1



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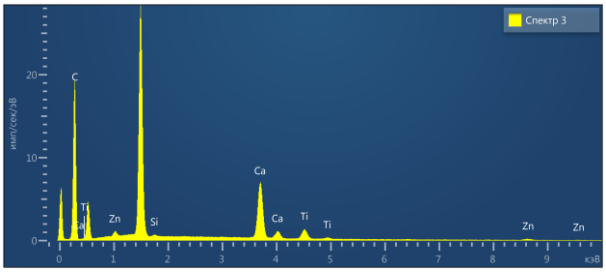
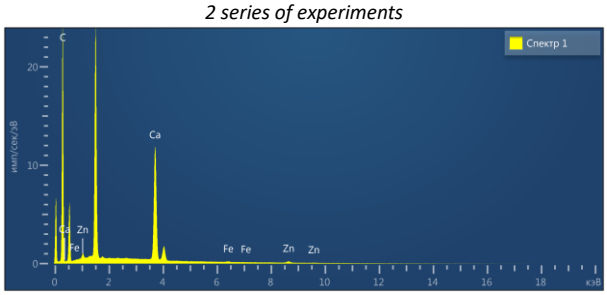


c) Sample 3

2 series of experiments



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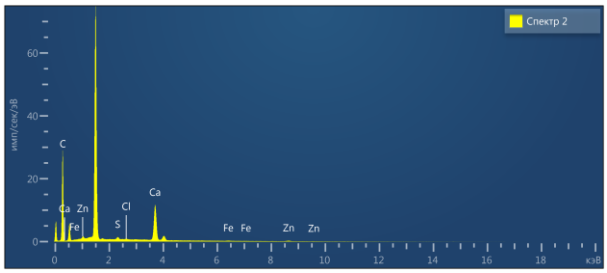

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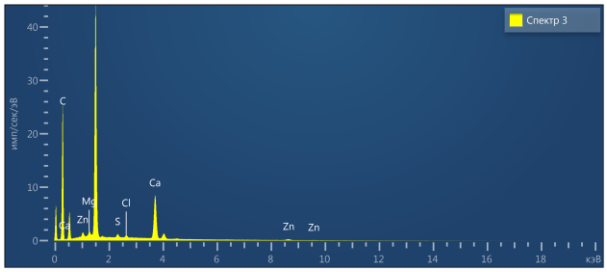

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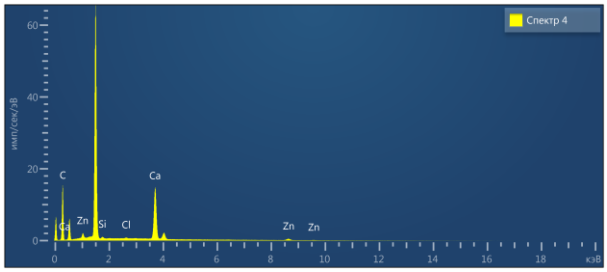
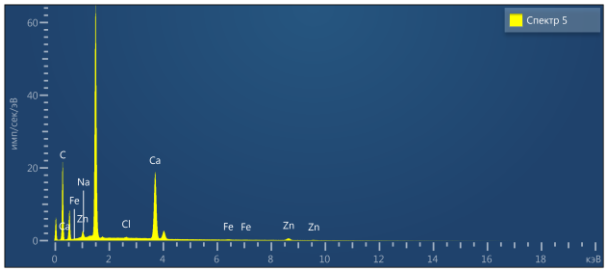
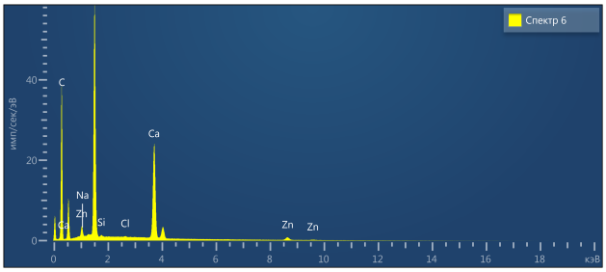


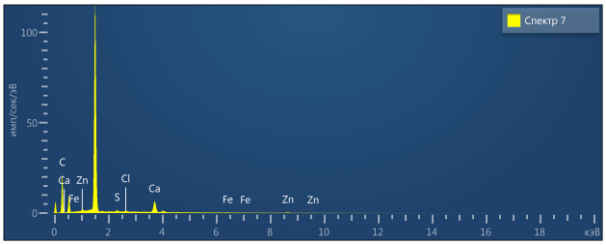
g) Sample 7



h) Sample 8



i) Sample 9



j) Sample 10

Figure 4. EDX spectrum of EVA material

Table 1. Spectral analysis of the chemical composition of the samples

Chemical element	Linetypes	wt. %	Sigma weight. %	Atom. %
Spectrum 1				
C	K- series	58.5	0.24	88.4
Ca	K- series	8.27	0.07	3.75
Ti	K- series	1.67	0.04	0.63
Zn	K- series	1.02	0.08	0.28
Br	L- series	30.53	0.19	6.93
Total		100		100
Spectrum 2				
C	K- series	76.02	0.29	91.82
Ca	K- series	20.02	0.19	7.25
Fe	K- series	1.32	0.14	0.34
Zn	K- series	2.64	0.25	0.59
Total		100		100
Spectrum 3				
C	K- series	72.72	0.27	90.44
Ca	K- series	18.7	0.17	6.97
Ti	K- series	5.09	0.12	1.59
Zn	K- series	2.83	0.22	0.65
Si	K- series	0.66	0.05	0.35
Total		100		100
Spectrum 4				
C	K- series	71.16	0.19	89.5
Ca	K- series	26.2	0.15	9.87
Zn	K- series	2.16	0.16	0.5
Fe	K- series	0.48	0.08	0.13
Total		100		100
Spectrum 5				
C	K- series	75.94	0.19	91.58
Ca	K- series	20.47	0.13	7.4
S	K- series	0.77	0.04	0.35
Zn	K- series	2.12	0.16	0.47
Fe	K- series	0.5	0.08	0.13

Chemical element	Linetypes	wt. %	Sigma weight. %	Atom. %
Cl	K- series	0.2	0.04	0.08
Total		100		100
Spectrum 6				
C	K- series	78.79	0.18	92.4
Ca	K- series	15.83	0.11	5.56
Mg	K- series	1.67	0.05	0.97
Zn	K- series	2.41	0.15	0.52
S	K- series	0.7	0.04	0.31
Cl	K- series	0.6	0.04	0.24
Total		100		100
Spectrum 7				
C	K- series	61.51	0.25	84.84
Ca	K- series	31.11	0.19	12.86
Zn	K- series	5.99	0.22	1.52
Cl	K- series	0.32	0.04	0.15
Si	K- series	1.07	0.04	0.63
Total		100		100
Spectrum 8				
C	K- series	62.2	0.21	85.27
Ca	K- series	30.31	0.16	12.45
Zn	K- series	6.08	0.17	1.53
Cl	K- series	0.28	0.03	0.13
Fe	K- series	0.46	0.08	0.14
Na	K- series	0.66	0.08	0.48
Total		100		100
Spectrum 9				
C	K- series	68.31	0.16	88.24
Ca	K- series	25.29	0.11	9.79
Zn	K- series	5.15	0.13	1.22
Si	K- series	0.53	0.02	0.29
Cl	K- series	0.16	0.02	0.07
Na	K- series	0.56	0.06	0.38
Total		100		100
Spectrum 10				
C	K- series	80.34	0.26	93.5
Ca	K- series	13.91	0.13	4.85
S	K- series	1.07	0.05	0.47
Cl	K- series	0.89	0.06	0.35
Zn	K- series	3.26	0.22	0.7
Fe	K- series	0.53	0.11	0.13
Total		100		100

Cocultivation of mesenchymal stem cells with polymeric materials showed no toxicity and satisfactory biocompatibility of all samples (Fig. 5).

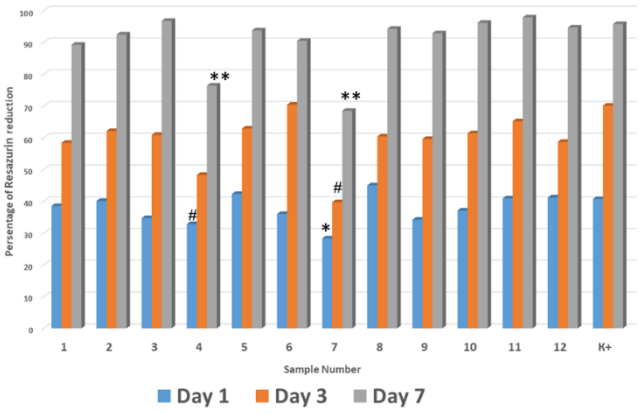
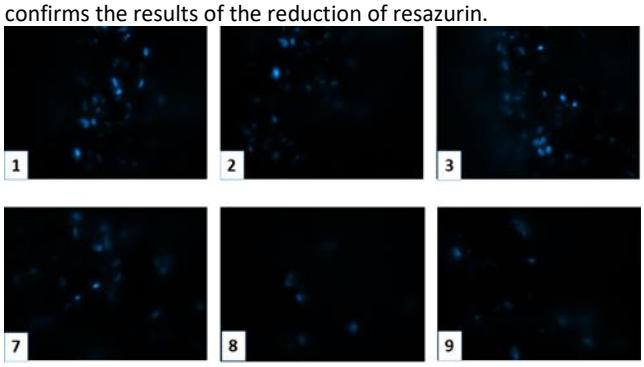


Figure 5. Diagram of the reduction of resazurin by MSC cells during cultivation with polymer samples for 7 days. * - significant difference (p≤0.05) with positive control on the 1st day; # - significant difference (p≤0.05) with positive control on the 3rd day; ** - significant difference (p≤0.05) with the positive control on the 7th day

One day after the start of the experiment, cells attach to the surface of the material, while we observed a significant difference in the percentage of resazurin reduction in comparison with the positive control on sample No. 7, which indicates the absence of satisfactory conditions for cell attachment. During the following observation periods, the proliferation of stem cells at the level of positive control occurs, and samples №4 and №7 are characterized by reduced reduction of resazurin. Nevertheless, the difference with the control does not exceed 20%, which allows to classify these materials as non-toxic.

Cell imaging on the last day of observation using DAPI-stained fluorescence microscopy showed a uniform distribution of MSCs on the surface of the polymer samples (Fig. 6).

Due to the uneven surface structure of the samples and pronounced porosity, we were unable to visualize 100% of the cells, but their location in the middle of the pores is also typical. As can be seen from Fig. 6 samples No. 4 and No. 7 are characterized by a smaller number of cells on the surface, which confirms the results of the reduction of resazurin.



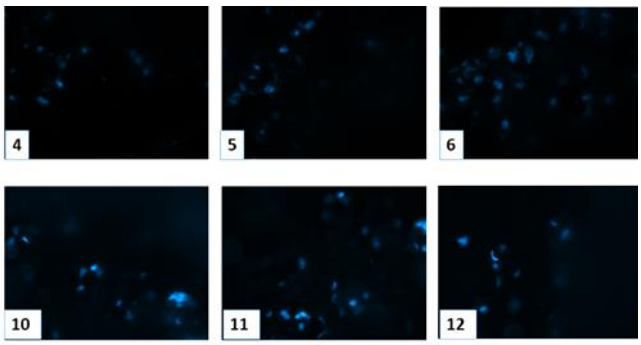


Figure 6. Fluorescence microscopy of the surface of polymeric samples on the 7th day after the start of the experiment. Color – DAPI.

Thus, the data obtained indicate the absence of toxicity and the presence of biocompatibility of polymer samples that are used to make orthopedic structures.

4. CONCLUSION

Medical device manufacturers should have qualitative and quantitative information about the characteristics of the final product. Such information can be obtained from the source material supplier from the literature or through additional testing. Product-specific information can be found in the relevant product standards. Determining and evaluating the physicochemical, morphological and topographic properties of materials used in the final medical device is important for evaluating the biological properties of products and materials from which they are made. The shape and geometrical dimensions of medical devices and their parts may affect the biological properties of the devices. The degree of assessment of material properties should reflect the nature and duration of clinical use and may also be of interest in evaluating the biosafety of the device. Such consideration of the characteristics is also important for the formation of a judgment on the equivalence of the proposed material used in the product with their biocompatibility and clinical efficacy. This assessment of the cytotoxicity of a mixture of polymers that have a closed-cell structure with a uniform distribution of cells over the surface will be further improved as more information becomes available in the development of orthopedic products.

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